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Membrane Cation and Anion Transport Activities in Erythrocytes of Hereditary Spherocytosis: Effects of Different Membrane Protein Defects

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Hereditary spherocytosis (HS) is due to different membrane protein defects (i.e., deficiency of spectrin and ankyrin, band 3, or band 4.2). In order to gain new insight into the relationships between band 3 function and proteins associated with the cytoskeleton, we studied erythrocyte anion transport activity in HS characterized by different membrane protein defects. Anion transport activity was increased in HS due to partial band 4.2 deficiency or to band 4.2 absence, while in HS associated with deficiency of spectrin + ankyrin or band 3, the anion transport results were normal or decreased, respectively. Moreover, since HS erythrocytes are characterized by an increased Na and a decreased K, we studied the principal membrane cation transport pathways. Activity of the Na/K pump was increased in all HS studied, while no changes in Na/K/2Cl cotransport and Na/Li exchange were evident between control and HS as well as between forms of HS associated with different membrane protein defects. K/Cl cotransport activity was decreased in all HS studied compared to normal red cells. In all HS, passive membrane permeability to Na and K was increased compared to normal erythrocytes. The increased Na and the low K content can be attributed to the abnormal membrane permeability to cations, which is not related to a specific membrane protein defect. Am. J. Hematol. 55:121–128, 1997. © 1997 Wiley-Liss, Inc.

Key words: erythrocytes; hereditary spherocytosis; anion transport; membrane cation permeability; band 4.2

INTRODUCTION

Hereditary spherocytosis (HS) is a common type of hereditary hemolytic anemia in Western countries, with a frequency of 1:5,000 births [1]. The disease is heterogeneous in terms of inheritance, red cell morphology, clinical severity, and underlying molecular defects [2]. The most frequent defect is spectrin deficiency [3,4] or combined deficiency of spectrin and ankyrin [5], but the primary molecular lesions have also been reported in band 3 and protein 4.2 [6].

Band 3 is the most abundant protein of the red cell membrane. The protein consists of two major domains with separate and largely independent functions [7,8]. The COOH-terminal ~55-KDa membrane domain mediates the anion exchange across the red cell membrane. The NH_2 terminal ~45-KDa cytoplasmic domain has the main function of anchoring the membrane skeleton to the membrane via interactions with ankyrin and protein 4.2 [9–11]. The erythrocyte membrane anion exchanger (band 3) allows the efflux of HCO_3^- from the cell in exchange for plasma Cl^- so as to equilibrate HCO_3^- between the red cells and plasma [9–11]. Anion transport activity has been described as altered in some HS [12,13]. Moreover, decreased anion transport activity in HS due to ankyrin deficiency has been shown, suggesting

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TABLE I. Hematological Data and Red Cell Cation Composition*

	Hct (%)	Hb (g/dl)	MCV (fl)	Retics (%)	Na (mmol/Kg Hb)	K (mm/Kg Hb)
Control ($n = 5$)	24.5 ± 0.9	7.1 ± 1.4	83.6 ± 3.6	5.7 ± 0.7	27.2 ± 2.3	287 ± 10
HS with band 3 deficiency ($n = 3$)	30.2 ± 2.5	9.2 ± 1.4	78.1 ± 8.5	6.1 ± 0.4	36.1 ± 2.7	225 ± 8.2
HS with Sp + Ank deficiency ($n = 4$)	39.5 ± 1.3	13.7 ± 0.9	76.7 ± 4.1	1.4 ± 0.6	39.2 ± 0.3	232 ± 11
HS with band 4.2 deficiency ($n = 4$)	28.6 ± 0.6	9.2 ± 0.7	80.5 ± 1.5	8.1 ± 1.9	38.1 ± 2.3	230 ± 6.1

*Data are presented as means ± SD (*n* of experiments).

that the proteins associated with the cytoskeleton may play a role as modulators of the transmembrane conformation of band 3 and may consequently affect anion transport activity [12,13].

Band 4.2 is another peripheral protein that binds the cytoplasmatic domain of band 3 and ankyrin [14]. Protein 4.2 and ankyrin bind to separate sites on the cytoplasmatic domain of band 3 [15–17]. Band 4.2 has homologies with transglutaminases, but it has no transglutaminase activity, so that the function of protein 4.2 in erythrocytes is still unknown [15–18]. The main evidence for an important role for the band 4.2 protein in red cells comes from studies in various forms of hereditary hemolytic anemias, in which erythrocytes are deficient in 4.2 protein [7,18].

HS erythrocytes are also characterized by increased Na and decreased K content compared to normal erythrocytes, in association with slight cell dehydration [19–23]. Recently, Joiner et al. [24] reported an increased red cell membrane permeability to monovalent cations in three mouse mutants as a consequence of deficiency in spectrin (sph/sph, sph^{ha}/sph^{ha}) or ankyrin (nb/nb). The authors suggested that the cytoskeleton dysfunction resulting from such membrane protein defects may also be responsible for the abnormal cation permeability of the red cell membrane [24]. Moreover, previous studies of HS erythrocytes were not able to discriminate between the presence of abnormalities in cation transport pathways and/or in membrane permeability to cation [19,23].

In order to verify if the perturbation of the red cell membrane skeleton due to different membrane protein defects might be responsible for changes in activity of the main cation transport pathways, we evaluated the function of the Na/K pump, Na/Li exchange, Na/K/2Cl cotransport, K/Cl cotransport, and membrane passive permeability to Na and K in red cells of HS associated with different membrane protein defects. In the same group of HS, we also studied the activity of the anion exchanger in order to evaluate the effect of different membrane protein defects on the anion exchanger function.

MATERIALS AND METHODS

Drugs and Chemicals

NaCl, KCl, $NaNO_3$, albumin (bovine fraction V), Tris (hydroxymethyl) aminomethane (Tris), 3-(N-morphilino) propanesulfonic acid (MOPS), ouabain, bumetanide, sucrose, glucose, and di-isothiocyano-dhydrostilbene disulphonate (H_2DIDS) were purchased from Sigma Chemical Co. $MgCl_2$, Mg_2NO_3, Na citrate, EGTA, and dimethylsulfoxide (DMSO) were purchased from Fisher Scientific Co. (^{35}S) sulfate was purchased from DuPont. All solutions were prepared using double-distilled water.

Patients

Informed consent was obtained from control subjects and patients. Blood was collected, after overnight fasting, into heparinized tubes and processed within 24 hr. We studied 10 patients with HS with a typical clinical form, characterized by incompletely compensated hemolysis with mild to moderate anemia, only sporadically requiring blood transfusion [25,26]. For all patients, diagnosis of HS was confirmed by a positive pink test. HS patients were divided into three groups, according to the biochemical alteration observed on SDS-PAGE analysis: group 1, band 3 deficiency (n = 3); group 2, combined spectrin (Sp) and ankirin (Ank) deficiency (n = 4); and group 3, isolated band 4.2 deficiency (n = 4); one of the subject had band 4.2 absence [25,26]. As normal controls (n = 5), we selected post traumatic hemorrhagic subjects without HS or other hematological diseases, with values of reticulocytes similar to those observed in HS patients. Hematological data of the patients are summarized in Table I.

Determination of Membrane Protein Composition

Freshly drawn blood anticoagulated in acid citrate/dextrose was treated within 48 hr of phlebotomy; erythrocyte ghosts were prepared by method of Dodge et al. with minor modifications described in Iolascon et al [26]. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) both in 3.5–

17% exponential gradient gels (Fairbanks method) and in 5–15% linear gradient gels (Laemmli method), as previously reported [25]. Gels were stained with Coomassie Brilliant Blue and, after destaining, were scanned using a laser-densitometer (Ultroscan, LKB) [25,26]. Particularly, amounts of the major membrane proteins were expressed as the value of the relative surface area present under the densitometric curve. Erythrocyte membrane analysis of each patient was performed three times utilizing different gels; we observed a reproducibility of spectrin and ankyrin measurements within a range of ±5%, whereas reproducibility of band 3 and protein 4.2 evaluation was within a range of ±4%. Amounts of each protein were compared to normal values obtained from controls, as previously reported [25,26].

Sulfate Influx Studies

Red cells were washed three times in 84 mM sodium citrate, 1 mM EGTA, pH 6.5. The influx of (^{35}S) sulfate was measured using equal numbers of HS and control red cells (determined using a cell counter) [27,29]. The flux was measured in a medium containing 4 mM sodium sulfate, 84 mM sodium citrate, and 1 mM EGTA, pH 6.5. Influx was measured after 10 min at 30°C in the presence of increasing concentrations of the anion transport inhibitor di-isothiocyano-dhydrostilbene disulphonate (H_2DIDS) [27–29]. Maximum inhibitory H_2DIDS concentration was calculated by least-squares linear regression analysis, using Systat 3.1 software [27]. For each determination, a control was measured in parallel. The sulfate flux was expressed for a constant number of cells (1×10^{13} cells), to allow comparison among patients with variable MCV values [27]. Since the maximal inhibitory H_2DIDS concentration gives the concentration of band 3 in the sample used, the data were expressed as the ratio between sulfate flux and maximal inhibitory H_2DIDS concentration. This ratio gives a measure of the relative transport activity per molecule of band 3, and it does not allow for changes in cell shape, size, or density of membrane proteins in the red cell membrane [27].

Measurements of Membrane Cation Transports in HS Red Cells

Erythrocyte Na and K content was determined on fresh cells after five washes in a washing solution (CWS) containing 152 mM choline chloride, 1 mM $MgCl_2$, and 10 mM Tris(hydroxymethyl)aminomethane-3(N-morpholino) propanesulphonic acid (Tris-MOPS). An aliquot of cells was suspended in approximately equal volumes of choline washing solution, and from the cell suspension determinations of hematocrit, cellular Na (using a 1:50 diluition in 0.02% acationix), and cellular K (using a 1:500 diluition in 0.02% acationix) were carried out. The erythrocyte Na and K contents were determined by atomic absorption spectrophotometry using standards in double-distilled water [30–33].

The maximal rates of Na/K pump activity were measured in cells containing equal amounts of Na and K (nystatin technique) [30–34]. With this procedure the internal sites for the Na/K pump are saturated. The nystatin-loading solution contained 70 mM NaCl, 70 mM KCl, and 55 mM sucrose. The Na/K pump was estimated as the ouabain-sensitive fraction of Na efflux into a medium containing 130 mM choline chloride and 10 mM KCl. Triplicate samples were incubated for 5 min and 25 min at 37°C. The hematocrit of the cell suspension was 1% and the ouabain concentration was 0.01 mM. The Na/K/2Cl cotransport (cot) was estimated as the bumetanide-sensitive fraction of the Na and K efflux into media containing 140 mM choline chloride and 0.1 mM ouabain. The efflux times were 5 min and 25 min at 37°C with triplicate samples. The bumetanide concentration was 0.01 mM. All media contained 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Tris-MOPS (pH 7.4 at 37°C) [30–33].

The Na/Li exchange was estimated as the external Na-stimulated Li efflux (difference between Li efflux into 140 mM NaCl and 140 mM choline Cl) from cells containing known amounts of Li (nystatin technique) [30–33].

The K/Cl cotransport (cot) from fresh cells was measured as the chloride-dependent K efflux and volume-dependent K efflux [26–28]. Flux media for chloride-dependent K efflux contained 100 mM Na and 1 mM Mg (the anion being either Cl and NO_3), with 10 mM Tris-MOPS (pH 7.4 at 37°C). Volume-dependent K fluxes were calculated as the differences between K efflux in NaCl hypotonic (100 mM) medium and that in NaCl isotonic (140 mM) medium. Efflux was calculated from the K loss at 5 and 25 min [30–33]. The membrane passive permeability to Na and K was evaluated as ouabain-bumetanide-resistant fluxes from fresh cells in isotonic medium [30–33].

RESULTS

Protein Composition of Red Blood Cell Membranes

Characteristics of the patients studied are presented in Table I. Three HS subjects showing band 3 deficiency, 4 patients with spectrin deficiency combined with ankyrin reduction, and 3 patients with partial isolated protein 4.2 reduction were studied (Fig. 1). The HS were divided into three groups, according to the observed membrane protein deficiencies. The extent of the membrane protein defect was similar in all groups, ranging from 20–30%.

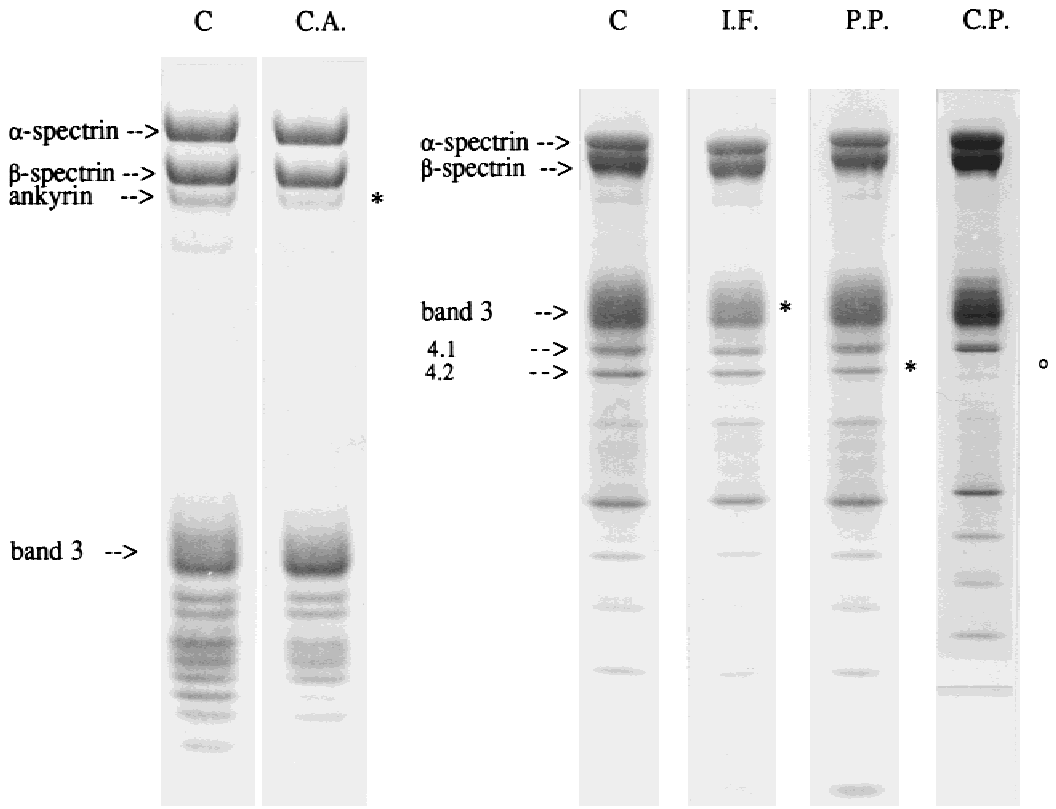

Fig. 1. SDS-PAGE performed both in 3.5–17% exponential gradient gels with the method of Fairbanks (left) and in 5–15% linear gradient gels according to Laemmli (right). *Proteins showing meaningful reduction. °Complete absence of band 4.2. Lane C, normal control. Lanes C.A., P.P., I.F., and C.P. represent subjects affected by HS with Sp + Ank, band 3 or band 4.2 deficiency, and band 4.2 absence, respectively.

Anion Transport in HS Erythrocytes

In band 3 deficiency, we observed a 15.3–38.5% decrease in the H_2DIDS-sensitive sulfate influx; in addition, a lower concentration of the anion transport inhibitor H_2DIDS was needed for maximal inhibition of sulfate influx, indicating a decreased number of H_2DIDS target (binding) sites (Table II). In combined spectrin + ankyrin deficiency, the H_2DIDS-sensitive sulfate influx and the maximal inhibitory H_2DIDS concentration were unaffected compared with controls (Table II). In partial isolated band 4.2 deficiency and in band 4.2 absence, we observed a marked increase in H_2DIDS-sensitive sulfate influx, while no remarkable changes in maximal inhibitory H_2DIDS concentration were evident between control and HS band 4.2 deficiency or HS with band 4.2 absence (Table II, Fig. 2). Since the maximal inhibitory H_2DIDS concentration gives the concentration of band 3 in the sample used, the data were also expressed as the ratio between sulfate flux and maximal inhibitory H_2DIDS concentration. This ratio gives a measure of the relative transport activity per molecule of band 3 [27], and it does not allow for changes in cell shape, size, or density of membrane proteins in the red cell membrane. As shown in Table II, the ratio for HS associated with band 3 deficiency and with Sp + Ank defect was very similar to that observed in normal controls. For HS with partial isolated band 4.2 deficiency and with band 4.2 absence, the ratio was respectively twofold and fourfold higher, suggesting an increase in activity of the anion transport for band 3 molecules (Table II).

Cation Transport Pathways in HS Erythrocytes

The HS erythrocytes showed lower K and higher Na content compared to control erythrocytes (Table I). We carried out experiments to study the activity of the Na/K pump, Na/K/2Cl cot, Na/Li exchange, and K/Cl cot in HS associated with different primary membrane protein defects. Because some of these transports are mostly expressed in young erythrocytes, we used as a control group normal subjects with reticulocyte values similar to those observed in HS patients.

As shown in Table III, activity of the Na/K pump was markedly increased in erythrocytes of HS compared to normal controls, but no differences were evident in the magnitude of Na efflux between the forms of HS associated with different membrane protein defects. The activity of Na/K/2Cl cot and of Na/Li exchange was similar to that observed in normal controls for all HS studied; no

TABLE II. Anion Exchange Activity in HS Erythrocytes*

Patient	Maximal sulfate influx (mmol sulfate/10^{13} cells × hr)	Maximal inhibitory [H_2 DIDS] (μM)	Ratio
Control			
M.P.	11.4	2.5	4.56
F.C.	12.3	2.8	4.39
P.T.	14.1	3	4.72
R.B.	13.4	2.9	4.62
B.P.	11.7	2.7	4.33
HS with band 3 deficiency			
G.L.	7.3	1.8	4.05
I.F.	6.8	1.5	4.53
G.S.	7.2	1.7	4.23
HS with Sp + Ank deficiency			
C.G.	10.4	2.3	4.52
C.A.	11.2	2.4	4.66
C.S.	13.7	3	4.56
L.M.	11.3	2.7	4.18
HS with band 4.2 deficiency			
P.P.	24.1	2.5	9.64
F.A.	20.3	2	10.15
C.M.	19.8	2.3	8.61
P.C.	47.4	2.3	20.6

*Fluxes are means of at least three triplicate samples. For each measurement, a normal control was evaluated in parallel. Ratio: maximal SO^{35} flux/maximal inhinbitory H_2DIDS concentration. H_2DIDS, at the concentrations used, binds band 3 in a stoichiometric way 1:1, so that the ratio represents the relative transport activity per molecule of band 3.

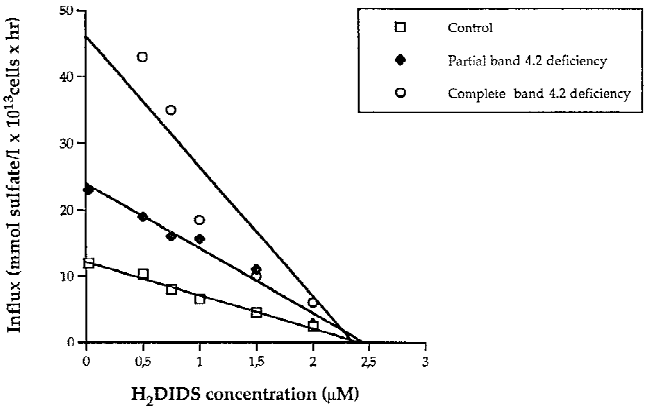

Fig. 2. H_2DIDS inhibition of sulfate flux: effect of increasing concentrations of H_2DIDS on [^{35}S] sulfate flux into red cells of patients with HS due to partial band 4.2 deficiency and complete band 4.2 deficiency, as compared with controls. Curves are representative of those observed in red cells of 2 other patients with a partial band 4.2 defect.

abnormalities in the function of these transports were detected between HS with different membrane protein defects (Table III).

In all HS studied, the K/Cl cot activity was lower compared to that of normal erythrocytes, indicating that the cell K loss characterizing HS red cells was not due to increased activity of this transport system (Table III).

The passive membrane permeability to Na and K in fresh erythrocytes was increased in all HS cells compared to normal red cells, while no differences were evident between the HS studied (Table III).

DISCUSSION

Analysis of erythrocyte membrane proteins by SDS-PAGE allows detection of different protein alterations in HS (i.e., spectrin and ankyrin, band 3, or band 4.2) [25,26]. The molecular basis of band 3 and of ankyrin reduction has been intensively investigated, and a large number of mutations determining these deficiencies have already been recognized in the gene encoding band 3 and band 4.2 [2,4]. The interpretation of the cases showing a 20% protein 4.2 reduction is problematic, since such reduction has been reported by ourselves and others as a secondary feature in some HS patients with primitive ankyrin or band 3 deficiency [3,7,25,26,35]. In HS associated with band 4.2 deficiency studied by SDS-PAGE analysis, there were no detectable combined alterations of other membrane proteins (Fig. 1).

In HS due to partial or complete band 4.2 deficiency, we observed for the first time increased activity of anion transport per molecule of band 3 (Table II, Fig. 2). Because the number of copies of band 4.2 on the erythrocyte membrane equals or exceeds that of the major structural proteins of the membrane skeleton, it is likely that band 4.2 plays an important role in membrane function or structure [14]. Recently, Malik et al. [36] studied, in vitro, the effect on anion transport activity of increasing

TABLE III. Red Cell Membrane Cation Transport Pathways in HS Associated With Different Membrane Protein Defects*

	Control (n = 5)	HS band 3 (n = 3)	HS Ank + Sp (n = 4)	HS band 4.2 (n = 4)
Na/K pump				
Ouabain-sensitive Na efflux	15.2 ± 1.9	23.2 ± 5.1	21 ± 2.4	21.4 ± 1.5
Na/K/2Cl cot				
Bumetanide-sensitive Na efflux	3.6 ± 0.8	3.8 ± 0.4	4.1 ± 0.7	3.9 ± 0.3
Bumetanide-sensitive K efflux	4.5 ± 0.7	4.7 ± 0.5	4.2 ± 0.7	4.1 ± 0.3
Na/Li exchange	0.6 ± 0.2	0.6 ± 0.1	0.7 ± 0.3	0.5 ± 0.3
K/Cl cot				
Volume-chloride K efflux	6.5 ± 1.2	3.5 ± 0.9	2.6 ± 0.7	3.6 ± 0.3
Membrane passive permeability				
Na influx	15.4 ± 2.7	34.7 ± 1.1	29.1 ± 3.2	33.1 ± 4.1
K efflux	2.1 ± 0.2	6.5 ± 0.7	5.9 ± 1.2	7.2 ± 2.3

*Na and K efflux data are expressed in units of mmol/Kg Hb × hr. Data are presented as means ± SD (*n* of experiments). Membrane passive permeability to Na and K was measured in the presence of 0.1 mM ouabain and 0.01 mM bumetanide.

amounts of band 4.2 complexed with purified band 3 reconstituted into liposomes. Using this model, the authors showed an inverse correlation between sulfate flux and band 4.2 protein content. According to the working model of band 3 proposed by Krupka [37,38], the altered function of the anion transporter may be interpreted as a decrease in the activation energy or as a structural change in the protein during the translocation step, or as an increase in stability of the transition state intermediate. It is possible that band 4.2, through its interaction with the band 3 cytoplasmatic domain, is keeping the transmembrane domain in a particular conformation that is not the most favorable for maximal anion transport [37,38]. A similar mechanism of modulation occurs when hemoglobin binds to the cytoplasmatic domain of band 3: binding of hemoglobin determines kinetic changes in anion transport activity, suggesting that conformational changes in the cytoplasmatic domain influence the transmembrane domain and modulate the function of the anion transporter.

A reduction in activity of the anion transport has already been reported in some of the HS due to band 3 deficiency [12,13]. The decrease of the number of band 3 molecules present in the erythrocyte membrane may be reflected by a decrease in the rate of transmembrane sulfate flux and by a decrease in the number of H_2DIDS-inhibitable sulfate transporters [27]. However, when anion transport function is expressed as activity of the transport per molecule of band 3, the HS associated with band 3 deficiency showed an anion transport activity similar to that observed in normal controls (Table II). Although the decrease in band 3 is frequently associated with a slight reduction in band 4.2, the anion transport function per molecule of band 3 did not seem to be affected when both membrane proteins were defective, suggesting that a normal band 4.2/3 molecular ratio is crucial in keeping the transmembrane domain in a conformation which permits normal function of the anion transporter.

As previously shown [19–23], we found that in HS the erythrocyte Na content was increased and the K content was reduced (Table I). In the HS studied, the function of the Na/K pump was increased compared to normal control red cells (Table III), while the activity of Na/K/2Cl cot and Na/Li exchange was similar to that observed in control erythrocytes, indicating that these membrane cation transports are not responsible for the abnormal red cell cation content. The K/Cl cot was reduced compared to control red cells, and the different membrane protein defects did not seem to influence the degree of inhibition of this transport, suggesting that the mechanism(s) common to the different forms of HS studied negatively interferes with K/Cl cot function. Reduction of transport may be related to aspecific membrane loss and the cellular fragmentation characterizing HS erythrocytes, as well as to defective skeleton membrane attachment, which may alter the functional structure of the transporter [2,4,25].

We conclude that the abnormalities in Na and K red cell content are not related to abnormalities of specific principal cation transport pathways, till now identified on red cell membranes. The increased membrane passive permeability to cations was similar in all HS studied and was not dependent on a specific membrane protein defect, suggesting that cytoskeleton dysfunction per se may alter the permeability barrier of the red cell membrane. This interpretation is supported by the increased cation membrane permeability reported in spherocytic mouse erythrocytes associated with spectrin or ankyrin deficiency, as well as by the alterations in membrane permeability observed in normal human red cells subjected to shear stress [24,39,40].

These data demonstrate that HS associated with band 4.2 deficiency or with band 4.2 absence has increased

anion transport activity. This finding is consistent with an earlier proposal, based on experiments with preconstituted liposomes, that band 4.2 may act as a negative modulator of band 3-mediated anion transport (36). Finally, we showed that increased Na and low K content, which characterize HS erythrocytes, cannot be attributed to abnormalities in the main cation transport pathways but to an alteration of membrane permeability, probably as a consequence of perturbation of cytoskeleton integrity which was not related to one specific membrane protein deficiency.

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